A DISSERTATION

FOR THE DEGREE OF MASTER PROGRAM

Induction of Heat Shock Proteins Enhances the Biological Characteristics of Canine Mesenchymal Stromal Cells in Fresh and Post-Thaw Cultures

Heat shock protein 활성화 처리가 신선 및 동결 후 개 중간엽 줄기 세포의 생물학적 특성에 미치는 증진 효과

by

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MAJOR IN VETERINARY SURGERY

DEPARTMENT OF VETERINARY MEDICINE

GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

August 2019

Induction of Heat Shock Proteins Enhances the Biological Characteristics of Canine Mesenchymal Stromal Cells in Fresh and Post-Thaw Cultures

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A Dissertation submitted to the

Graduate School of Seoul National University

in partial fulfillment of the requirement for the degree of

Master in Veterinary Surgery

May 2019

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Submitting a Master thesis of Veterinary Surgery

April 2019

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Abstract

Allogenic, adipose-derived mesenchymal stromal cells (Ad-MSCs) are a good resource for cytotherapy owing to their antioxidant and anti-inflammatory properties. Although frozen then thawed allogenic Ad-MSCs can be used instantly for this purpose, cryopreservation is detrimental to Ad-MSCs, particularly in terms of cell viability and antioxidant capacity. Therefore, in vitro procedures should be developed to improve post-thaw stromal cell characteristics. In the present study, freshly cultured Ad-MSCs were activated with heat shock, hypoxia (5% O_2), or hypoxia (5% O_2) + heat shock treatments. The results showed that, compared to the other treatments, heat shock significantly improved overall stromal cell characteristics related to antioxidant capacity, heat shock proteins, growth factors, and proliferation rate. Consequently, stromal cells were heat shocked and then treated with different combinations of dimethyl sulfoxide and fetal bovine serum (FBS) to determine the

proper combination that effectively preserves the characteristics of stromal cells after cryopreservation. I found that heat shock-preconditioned stromal cells cryopreserved with 10% dimethyl sulfoxide (Me_2SO) + 40% fetal bovine serum (FBS) presented significantly ($p < 0.05$) improved cell viability, proliferation rate, antioxidant capacity, and stemness, as well as significantly higher levels of heat shock proteins, growth factors, and anti-inflammatory factors, compared to cryopreservation with 1% Me_2SO + 10% FBS or 1% Me_2SO alone. I concluded that heat shock treatment can enhance the desired characteristics of stromal cells in both fresh and post-thaw cultures.

Keywords: Heat shock treatment, Hypoxia, Cryopreservation

Student number: 2017-21476

Contents

List of Abbreviations

Ad-MSCs	Adipose Derived Mesenchymal Stromal Cells
FBS	Fetal Bovine Serum
HST	Heat Shock Treatment
HT	Hypoxia Treatment (5% O_2)
HT+HST	Hypoxia Treatment + Heat Shock Treatment
HST-Ad-MSCs	Heat Shock Treated Ad-MSCs
HT-Ad-MSCs	Hypoxia Treated Ad-MSCs
HT+HST-Ad-MSCs	Hypoxia Treated + Heat Shock Treated Ad-MSCs
Me_2SO	Dimethyl Sulfoxide
PBS	Phosphate Buffer Saline
SOX-2	SRY (sex determining region Y)-box 2
OCT-4	Octamer-Binding Transcription factor 4
NANOG	Homeobox Protein NANOG

HSP-27	Heat Shock Protein 27
HSP-70	Heat Shock Protein 70
COX-2	Cyclooxygenase 2
IL-6	Interleukin 6
Il-10	Interleukin 10
VEGF	Vascular Endothelial Growth Factor
HGF	Hepatocyte Growth Factor
CXCR-4	Chemokine Receptor Type 4
CXCL-12	Stromal Cell-Derived Factor 1
MCP-3	Monocyte Receptor 3

Introduction

Adipose-derived mesenchymal stem cells are a well-established resource for tissue engineering and regenerative medicine applications. Stem cells are self-renewing and multipotent and possess the ability to secrete cytokines and chemokines (Gimble, Katz, & Bunnell, 2007; Zuk, 2013); therefore, they can be used in cell-based therapies to treat diseases of the bone (Barba, Cicione, Bernardini, Michetti, & Lattanzi, 2013), heart (Qayyum et al., 2012), kidney (Donizetti-Oliveira et al., 2012), and nervous system (Leu et al., 2010). However, long-term culture of stem cells has raised biosafety concerns, such as the occurrence of chromosomal aberrations and spontaneous malignant transformations (Froelich et al., 2013; Roemeling-van Rhijn et al., 2013). Therefore, it is necessary to be able to stably store stem cells for future use. Cryopreservation between −80 °C to −196 °C is the most commonly used method to store stem cells to halt cellular metabolism (Baust, Gao, & Baust 2009). If properly preserved, autologous and allogeneic stem cells can be used for clinical purposes, either directly from fresh culture, or following cryopreservation. Dimethyl sulfoxide (Me_2SO) is the most widely used stem cell cryoprotective agent (Shaik, Hayes, Gimble, & Devireddy, 2017; Thirumala, Gimble, & Devireddy, 2010). Although convenient, cryopreservation for stem cell storage also reduces post-thaw cell viability (75–85%) as a result of cryoinjury (Thirumala et al., 2010). Cryoprotectant toxicity against stem cells triggers apoptosis, the main cause of the low survival rate of cryopreserved cells (Heng et al., 2006). To overcome this, substantial research has been undertaken in the last decade to investigate and optimize stem cell cryopreservation protocols (Devireddy, 2014), and various treatments have been applied to stem cells to reduce the adverse effects of cryopreservation. For example, hypoxia-pretreatment enhances the post-thaw viability and proliferation rates of cryopreserved stem cells (Safwani et al., 2017). In contrast, heat shock treatment of stem cells, even when they are cryopreserved with a reduced amount of

cryoprotectants and without animal serum, enhances their post-thaw viability and stemness (Shaik et al., 2017) likely because increased levels of heat shock proteins can mitigate stress-related damage (Beere, 2005). So although hypoxia and heat shock treatments both contribute to increased viability and proliferation of cryopreserved stem cells, comparative effects of these treatments are still unclear. Therefore, I compared the effects and assessed the synergism between these two treatments on stromal cell characteristics. Moreover, I investigated the optimal concentration of fetal bovine serum (FBS) and Dimethyl sulfoxide (Me_2SO) in which HST-Ad-MSCs can retained most of their desired characteristics after cryopreservation. The aim of the present study was to investigate, in a fresh culture, the best treatment for improving stem cell desired characteristics among the following: heat shock treatment (HST), hypoxia (5% O_2) treatment (HT), and hypoxia (5% O_2) plus heat shock (HT+HST) treatment. Depending on the results, I then pretreated stromal cells with heat shock treatment and cryopreserved them with different compositions of Me_2SO and fetal bovine serum (FBS) to assess the minimum levels of cryoprotectants and serum that could yield the best results in terms of cell viability, proliferation rate, antioxidant capacity, stemness, anti-stress response, growth, and immunomodulatory properties.

Material and methods

Isolation of adipose-derived mesenchymal stromal cells

Canine adipose-derived mesenchymal stromal cells (Ad-MSCs) were isolated according to the method of Kisiel et al (Kisiel et al., 2012). I previously reported on the characterization and differentiation of Ad-MSCs isolated through this method (Khan, Yoon, Kim, & Kweon, 2017). Gluteal subcutaneous adipose tissue was collected aseptically from healthy male dogs aged 1–1.5 years. All animal experiments were approved by the Institute of Animal Care and Use Committee of Seoul National University (SNU-160720-13) and were carried out in

accordance with the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The adipose tissue was minced aseptically in a biosafety cabinet and then incubated with collagenase type 1A (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. The suspension was filtered through a 100 μm nylon mesh and centrifuged at 980 × *g* for 10 min. The supernatant was discarded and the pellet was resuspended in a culture medium. The cells were then cultured in DMEM (low glucose pyruvate, GIBCO BRL, Grand Island, NY, USA) with 10% FBS and 1% penicillin and streptomycin (PS, 10000 U/mL, GIBCO). After 24 h, the cells were washed with PBS to remove tissue debris and floating cells. Fresh medium was then introduced and changed after every 48 h until 90% confluence. Confluent cells were either subcultured or cryopreserved. Ad-MSCs at the third passage were used for subsequent experiments.

Allocation of Ad-MSCs into groups

The Ad-MSCs were divided into 4 treatment groups: (1) **Control Ad-MSCs**, Ad-MSCs were cultured with 20% O_2 and 5% CO_2 at 37 °C until 90% confluence; (2) **HT-Ad-MSCs**, Ad-MSCs were cultured with 5% O_2 and 5% CO_2 at 37 °C until 90% confluence (Ahmed, Murakami, Kaneko, & Nakashima, 2016); (3) **HST-Ad-MSCs**, Ad-MSCs were cultured with 20% O_2 and 5% CO_2 at 37 °C until 90% confluence and then the culture plates were incubated for 1 h at 43 °C, with 20% O_2 and 5% CO_2. After 1 h of heat shock treatment, the culture plates were returned to the 37 °C cell culture incubator and incubated for an additional 3 h before further experimentation (Shaik et al., 2017); and (4) **HT+HST-Ad-MSCs**, Ad-MSCs were first cultured until 90% confluence under hypoxic conditions (5% oxygen). During hypoxia, the cells were subjected to heat shock treatment for 1 h at 43 °C followed by a 3 h incubation with 5% O_2 at 37 °C.

Cryopreservation

Since the HST-Ad-MSCs group showed the best overall results, I selected this group for the subsequent cryopreservation experiments. The Ad-MSCs and HST-Ad-MSCs were harvested at 90% confluence with 0.05% trypsin-EDTA (Sigma-Aldrich), then centrifuged at 2500 rpm for 5 min at 4 °C. The supernatant was discarded and 1×10^7 cells from each group were re-suspended in three different combinations of cryogenic medium: (1) 10% Me_2SO + 40% FBS; (2) 1% Me_2SO + 10% FBS; and (3) 1% Me_2SO only. The cells were transferred to cryovials (1.2 mL, Sigma-Aldrich), and then kept at 4 °C for 10 min. Using a cooling rate of 1 °C/min, the cryovials were cooled to −80 °C for 24 h and then kept at −150 °C for 1 week.

Cell morphology

In the fresh culture groups, the morphology of third passage stromal cells was observed at 4^{th} day of seeding after the respective treatments. The morphology of the post-thaw Ad-MSCs (third passage) was assessed on day 4 after seeding. Ad-MSCs were seeded in six-well plates at 1×10^5 Ad-MSCs per well. Cell morphology was observed with an EVOS XL Core cell imaging system (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Trypan blue exclusion and MTS assays

The viability of fresh and frozen-thawed stromal cells was determined by the trypan blue exclusion assay (Gibco) using a Countess II FL automated cell counter (Thermo Fisher Scientific). The proliferation rates of freshly cultured and post-thaw stromal cells were determined by the MTS cell proliferation assay, following the manufacturer's guidelines (Bio-vision, Milpitas, CA, USA). Approximately 5×10^3 cells were seeded per well of 96-well culture plates and proliferation rates were evaluated at 6, 24, 48, and 72 h after

seeding. A 20 µL aliquot of MTS reagent was added to each well and incubated for 2 h. The absorbance was measured at 490 nm using the Epoch Gen 5.2 type reader (Bio Tek, Winooski, VT, USA).

Total antioxidant capacity assay

The total antioxidant capacity (TAC) assay kit (CS0790, Sigma-Aldrich) was used, following the manufacturer's instructions (Tome, Baker, Powis, Payne, & Briehl, 2001). Approximately 1×10^7 cells from each group (fresh or cryopreserved) were centrifuged at 2500 rpm, washed twice in PBS, and re-suspended in the assay buffer of the kit. Cell lysates were prepared by four freeze-thaw cycles. The lysate mixtures were centrifuged at 15000 × *g* for 10 min and the supernatants were transferred to a separate tube. The lysates were mixed in a 96-well plate with a 1× myoglobin working solution (Sigma-Aldrich), an ABTS working solution from the kit, and 3% hydrogen peroxide (Sigma-Aldrich). The mixture was incubated at 25 °C for 5 min, and the reaction was stopped by adding stop solution from the kit to each well. The absorbance was read at 405 nm with an Epoch Gen 5.2 type reader (BioTek). The absorbance readings were transformed using a standard curve based on the soluble antioxidant Trolox. TAC is expressed as Trolox concentration (mM).

RNA isolation and real-time quantitative PCR (RT-qPCR)

Total mRNA was extracted from all groups before and after cryopreservation. For qPCR, mRNA was harvested using the Hybrid-R RNA extraction kit (GeneAll, Seoul, Republic of Korea) according to the manufacturer's guidelines. Total mRNA was determined by measuring the optical density at a wavelength of 260 nm using the Epoch Gen 5.2 type reader (BioTek). cDNA was synthesized using the PrimeScript II first strand cDNA synthesis kit (Takara, Otsu, Japan) and then amplified using the ABI StepOnePlus Real-

Time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) after mixing with SYBR Premix Ex Taq (Takara, Otsu, Japan) and the specified forward and reverse primers (Table 1). The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C (20 s), annealing at 59–61 °C (20 s), and extension at 72 °C (20 s). Gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), with GAPDH as the reference gene.

Sr	Target gene	Primer sequence
1.	***SOX2***	Forward: 5′-AACCCCAAGATGCACAACTC-3′
		Reverse: 5′-CGGGGCCGGTATTTATAATC-3′
2.	***NANOG***	Forward: 5′-GAATAACCCGAATTGGAGCAG-3′
		Reverse: 5′-AGCGATTCCTCTTCACAGTTG-3′
3.	***OCT-4***	Forward: 5′-GTCACCACTCTGGGCTCTCC-3′
		Reverse: 5′-TCCCCGAAACTCCCTGCCTC-3′
4.	***HSP-70***	Forward: 5′-ACATCAGCCAGAACAAGCGA-3′
		Reverse: 5′-GAAGTCGATGCCCTCGAACA-3′
5.	***HSP-27***	Forward: 5′-TAACTGGCAAGCACGAAGAG-3′
		Reverse: 5′-TCGAAGGTGACGGGAATAGT-3′
6.	***HO-1***	Forward: 5′-CCAGTGCCACGAAGTTCAA-3′
		Reverse: 5′-TCTTGTGCTCTGCTGCCAAC-3′
7.	***SOD-1***	Forward: 5′-AGTGGGCCTGTTGTGGTATC-3′
		Reverse: 5′-AGTCACATTGCCCAGGTCTC-3′
8.	***MCP-3***	Forward: 5′-GTGGCGGCCGCTCGAGATGAAGGTCTCCGCAGCG-3′
		Reverse: 5′-GCCCTCTAGACTCGAGTCACAGCTTTGGGGCTTGGG-3′

9.	***CXCR-4***	Forward: 5′-GAGCGGTTACCATGGAAGAG-3′
		Reverse: 5′-CGGTTGAAGTGAGCATTTTCC-3′
10.	***CXCL12***	Forward: 5′-GTGGCGGCCGCTCGAGATGAACGCCAAGGTCGCC-3′
		Reverse: 5′-GCCCTCTAGACTCGAGTTACTTGTTTAGAGCTTTCTCC-3′
11.	***COX-2***	Forward: 5′-ACCCGCCATTATCCTAATCC-3′
		Reverse: 5′-TCGGAGTTCTCCTGGCTTTA-3′
12.	***IL-6***	Forward: 5′-GGCTACTGCTTTCCCTACCC-3′
		Reverse: 5′-TTTTCTGCCAGTGCCTCTTT-3′
13.	***IL-10***	Forward: 5′-CCACGACCCAGACATCAAGAA-3′
		Reverse: 5′-TCCACCGCCTTGCTCTTATTC-3′
14.	***BDNF***	Forward: 5′-GCTGGCGGTTCATAAGGATA-3′
		Reverse: 5′-GTTTCCCTTCTGGTCATGGA-3′
15.	***VEGF***	Forward: 5′-CTATGGCAGGAGGAGAGCAC-3′
		Reverse: 5′-GCTGCAGGAAACTCATCTCC-3′
16.	***HGF***	Forward: 5′-ATGGGGAATGAGAATGCAG-3′
		Reverse: 5′-GACAAAAATGCCAGGACGAT-3′
17.	***PDGF-β***	Forward: 5′-CCCTGAGGGATGGTACTGAA-3′
		Reverse: 5′-GCAATGAGCACCGTACGTAGT-3′
18.	***GAPDH***	Forward: 5′-CATTGCCCTCAATGACCACT-3′
		Reverse: 5′-TCCTTGGAGGCCATGTAGAC-3′

Statistical analysis

The results are expressed as means ± SD. The data were analyzed using GraphPad Prism

software (version 5). The Kruskal–Wallis test with Mann–Whitney post-hoc test was used to assess differences between the groups. A *P*-value < 0.05 indicates a significant difference between the groups. All the experiments were repeated at least three times.

Results

Comparison of the characteristics of fresh Ad-MSCs treated with HST, HT, and HT+HST

Images of the fresh cultures from all the groups were acquired after the respective treatments at 4th day of seeding. All the cells from the fresh culture Ad-MSC groups were plastic-adherent and showed fibroblast-like morphology (Fig. 1).

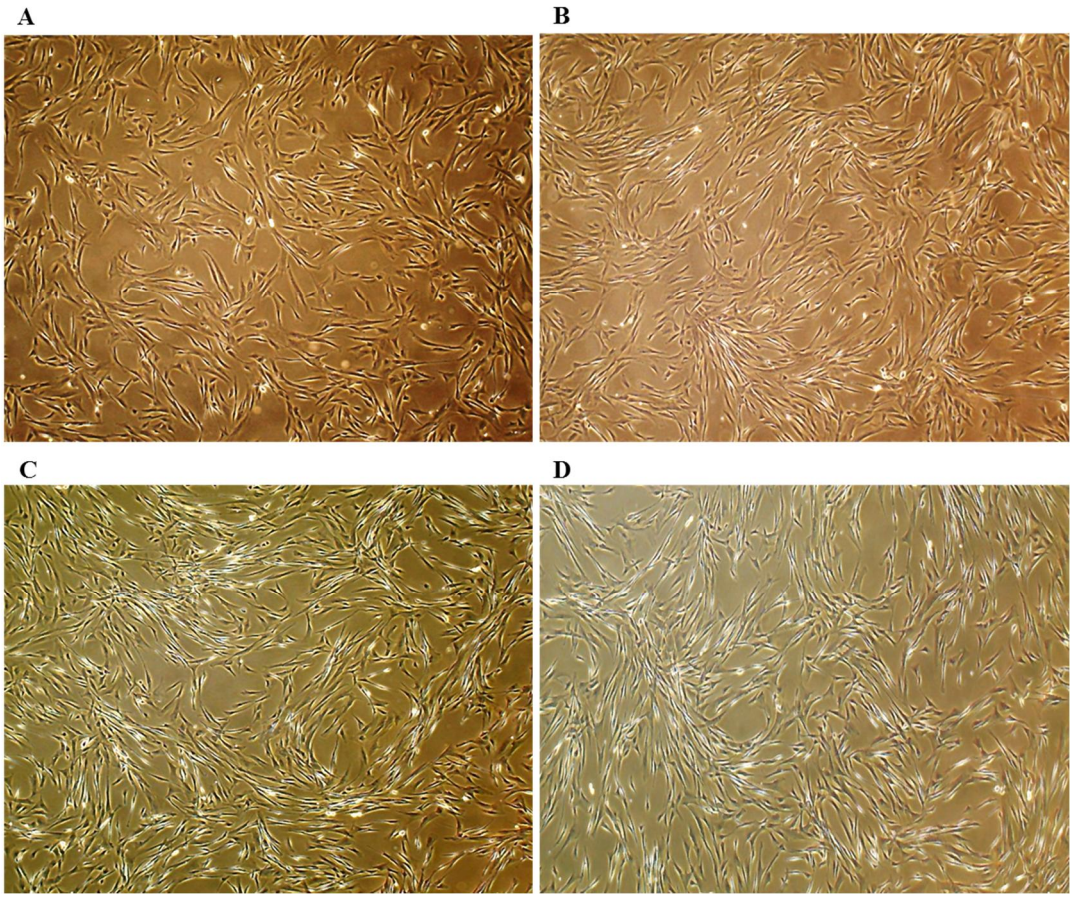

Figure 1 Morphology of fresh stromal cell groups. Fresh stromal cell cultures (third passage) from all

groups. (A) Control Ad-MSCs, (B) HT-Ad-MSCs, (C) HST-Ad-MSCs, and (D) HT + HST-Ad-MSCs after the respective treatments at 4th day of seeding, showing fibroblast-like cell morphology (×40 magnification).

The cell viability values for control Ad-MSCs, HT-Ad-MSCs, HST-Ad-MSCs, and HT + HST-Ad-MSCs were 90.6% ± 0.6%, 92% ± 0.6%, 91% ± 0.5%, and 90.7% ± 0.4%, respectively. There was no significant ($p > 0.05$) difference between groups (Fig. 2A). The proliferation rates of HT-Ad-MSCs and HST-Ad-MSCs were significantly ($p < 0.05$) higher than the control and HT + HST-Ad-MSC groups at 72 h after seeding (Fig. 2B). Among the fresh cultures, HST-treated Ad-MSCs showed significantly ($p < 0.05$) higher anti-oxidant capacity than the other groups (Fig. 2C).

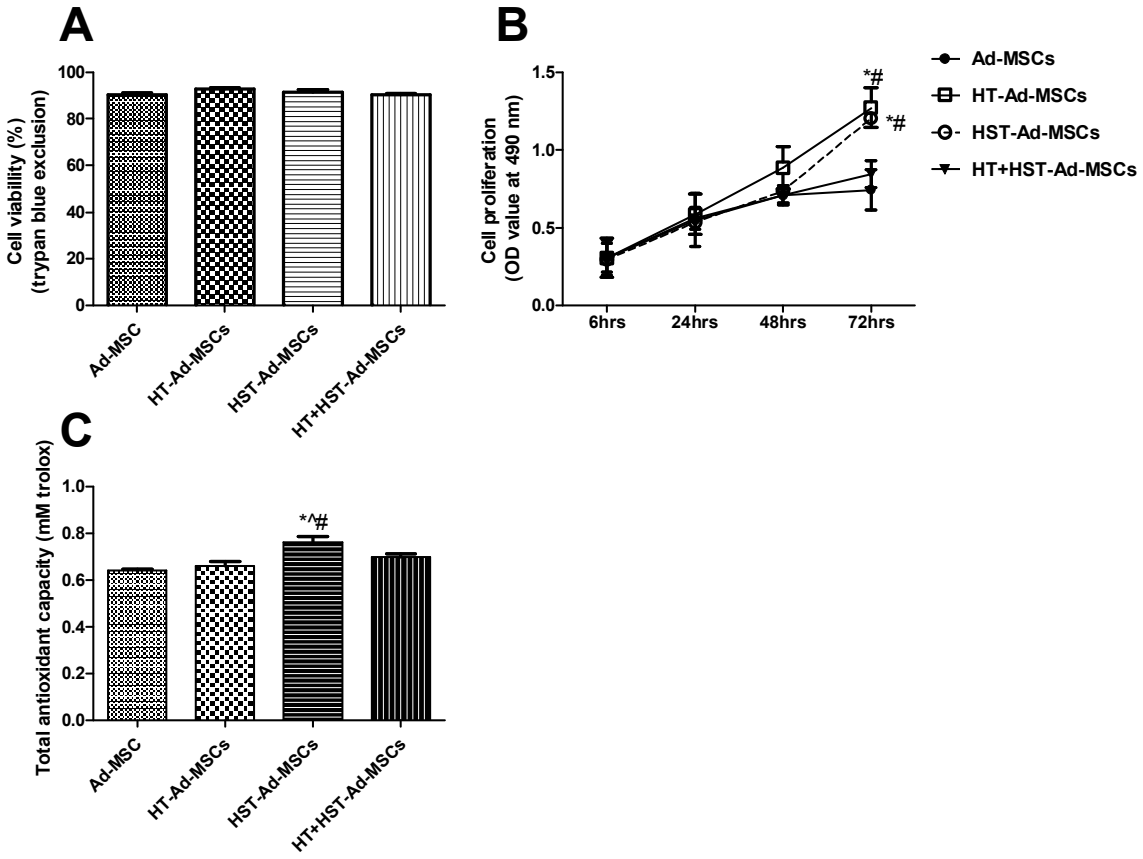

Figure 2 Viability, proliferation, and antioxidation capacity in fresh culture in relation to cell

activation treatments. (A) There was no significant ($p > 0.05$) difference in viability among all the groups. (B) Proliferation rates with HST-Ad-MSCs and HT-Ad-MSCs were both significantly ($p < 0.05$) higher than in the other groups. (C) The HST-Ad-MSC group showed significantly ($p < 0.05$) higher anti-oxidant capacity. * $p < 0.05$ vs control; ^ $p < 0.05$ vs the HT group; # $p < 0.05$ vs the HT + HST group.

The mRNA expression level of SRY (sex determining region Y)-box 2 (SOX2) in the freshly cultured HST group was significantly ($p < 0.05$) higher compared to the other groups (Fig. 3A). The octamer-binding transcription factor 4 (OCT-4) mRNA level was significantly ($p < 0.05$) higher in both the HST and HT groups compared to the other groups (Fig. 3B).

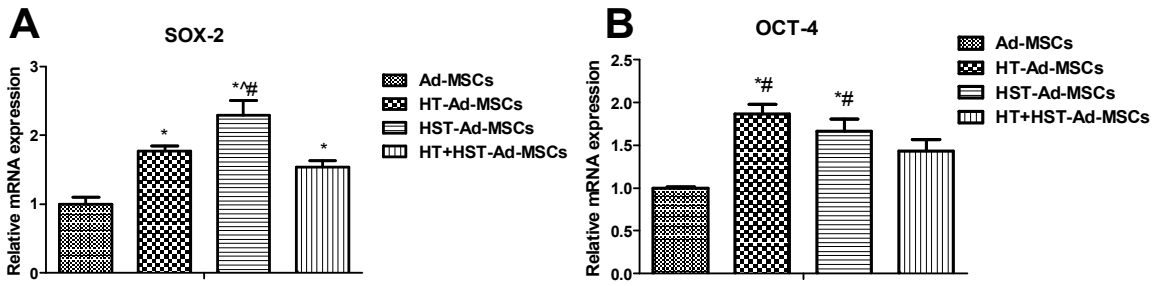

Figure 3 Stemness markers of fresh groups. (A) Fresh HST-Ad-MSCs had significantly ($p < 0.05$) higher SOX-2 compare to other groups (B) Fresh HST-Ad-MSCs had significantly ($p < 0.05$) higher OCT-4 mRNA expression levels compared to control and HT + HST groups. In the comparison of means, *denotes $p < 0.05$ compared to control, ^denotes $p < 0.05$ compared to HT, #denotes $p < 0.05$ compared to HT +HST.

Moreover, heat shock protein 27 (HSP-27) and 70 (HSP-70) were expressed at significantly ($p < 0.05$) higher levels in the HST-treated group (Fig. 4A and B). The HST- and HT + HST-treated Ad-MSC groups showed significantly ($p < 0.05$) higher heme oxygenase 1 (HO-1)

mRNA expression levels than the control and HT-treated Ad-MSC groups (Fig. 4C).

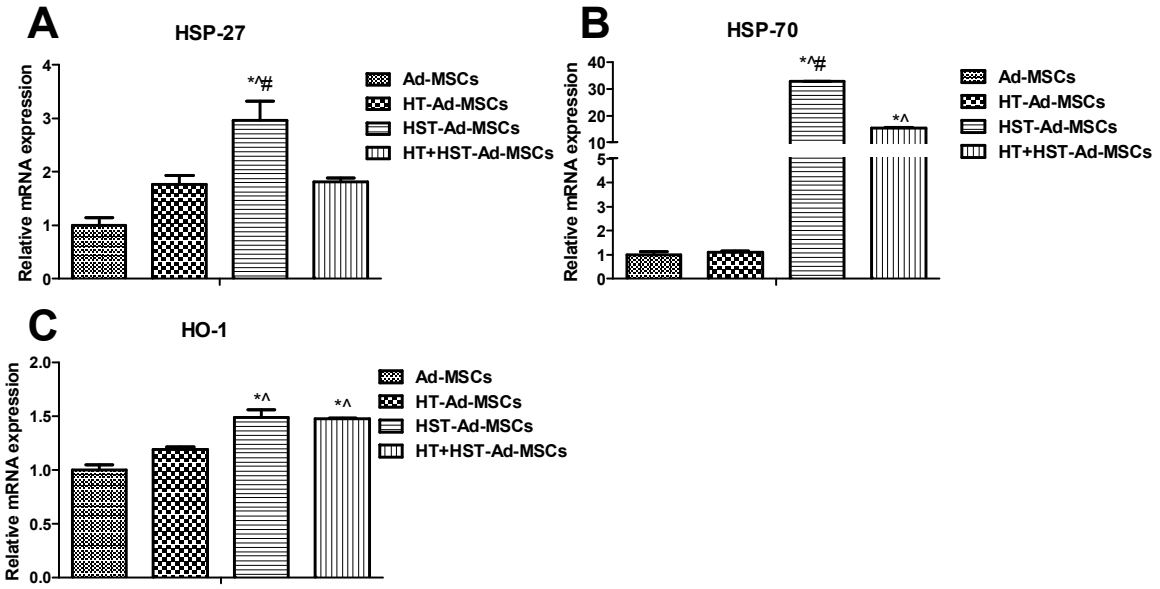

Figure 4 Heat shock proteins mRNA expression of fresh groups. (A) Fresh HST group had significantly ($p < 0.05$) higher mRNA levels of HSP-27 and (B) HSP-70 compared to the other groups. (C) The HST- and HT + HST-treated Ad-MSC groups had significantly ($p < 0.05$) higher *HO-1* mRNA expression levels compared to the other groups. *denotes $p < 0.05$ compared to control, ^denotes $p < 0.05$ compared to HT, #denotes $p < 0.05$ compared to HT +HST.

The mRNA expression levels of inflammatory factors like cyclooxygenase 2 (*COX-2*) and Interleukin 6 (*IL-6*) in all the groups did not differ significantly ($p > 0.05$) (Fig. 5A and B). However, the Interleukin 10 (*IL-10*) mRNA expression level in the HST group was significantly higher ($p < 0.05$) than in the other groups (Fig. 5C). These results suggest that HST treatment enhances the anti-inflammatory effects of Ad-MSCs.

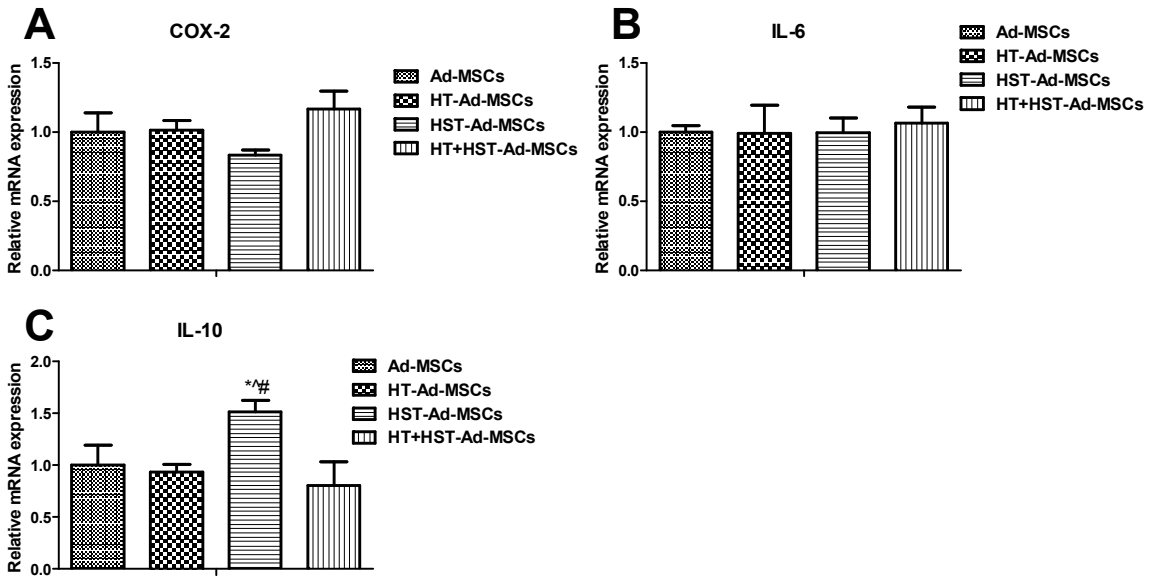

Figure 5 Immunomodulatory factors mRNA expression of fresh groups. (A) In fresh cultures, there was no significant ($p > 0.05$) difference in the levels of inflammatory *COX-2* and (B) *IL-6* between all groups. (C) *IL-10* was significantly ($p < 0.05$) higher in the fresh HST group than in the other groups. *denotes $p < 0.05$ compared to control, ^denotes $p < 0.05$ compared to HT, #denotes $p < 0.05$ compared to HT+HST.

The mRNA expression levels of vascular endothelial growth factor (*VEGF*) and hepatocyte growth factor (*HGF*) in HT- and HST-treated fresh cultures in the Ad-MSC groups were significantly ($p < 0.05$) higher compared to the control and HT + HST groups (Fig. 6A and B).

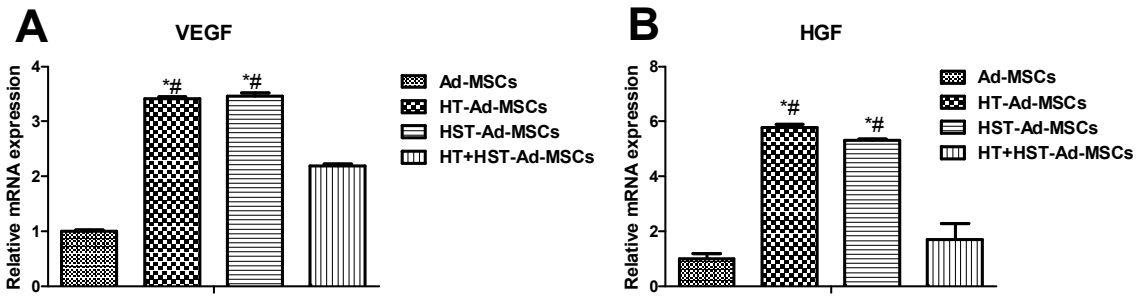

Figure 6 Growth factors mRNA expression of fresh groups. (A) Fresh HT and HST groups showed significantly ($p < 0.05$) higher mRNA expression levels of *VEGF* and (B) *HGF* compared to the other groups. *denotes $p < 0.05$ compared to control, ^denotes $p < 0.05$ compared to HT, #denotes $p < 0.05$ compared to HT + HST.

HST-Ad-MSCs showed significantly higher ($p < 0.05$) chemokine receptor type 4 (*CXCR-4*) and monocyte-chemotactic protein 3 (*MCP-3*) mRNA expression levels compared to the other groups (Fig. 7A and B). In contrast, stromal cell-derived factor 1 (*CXCL12*) mRNA expression was significantly higher ($p < 0.05$) only in HT-treated Ad-MSCs (Fig. 7C).

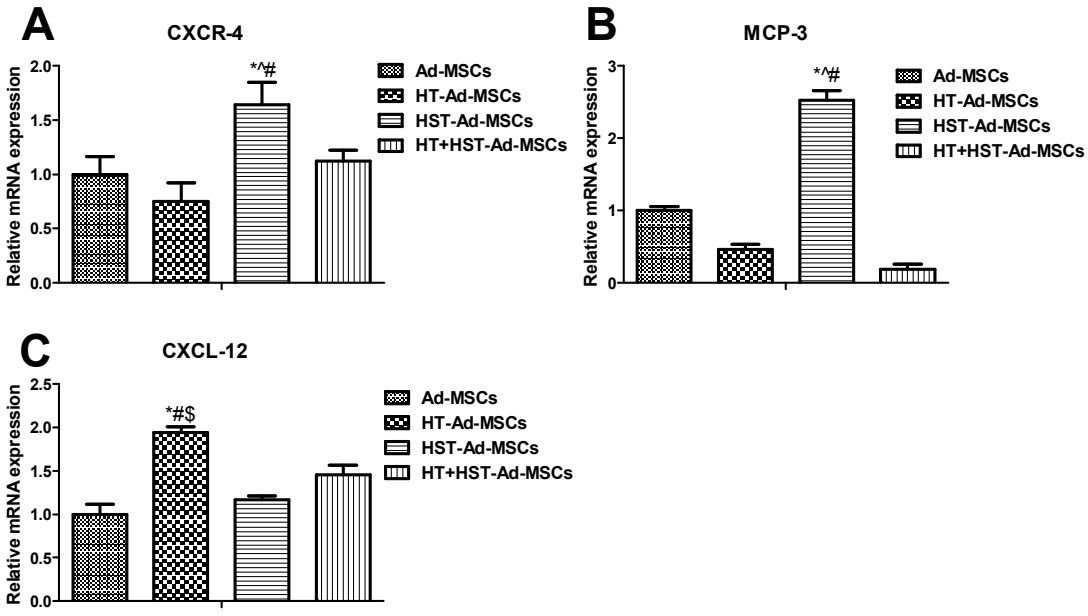

Figure 7 Homing factors mRNA expression of fresh groups. (A) Fresh HST-group had significantly

($p < 0.05$) higher mRNA levels of *CXCR-4* and (B) *MCP-3* compared to the other fresh groups. (C) Fresh HT-group presented a significantly ($p < 0.05$) higher level of *CXCL12* mRNA compared to the other groups. In the comparison of means, *denotes $p < 0.05$ compared to control, ^denotes $p < 0.05$ compared to HT, $ denotes $p < 0.05$ compared to HST, #denotes $p < 0.05$ compared to HT + HST.

Comparison of the characteristics of HST-Ad-MSCs cryopreserved with different combinations of Me_2SO and FBS

Images of post-thaw cultures were acquired 4 d after seeding. The growth of all HST groups was significantly ($p < 0.05$) higher than their respective control groups. Moreover, HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS showed the highest growth as compared to all other cryopreserved groups (Fig. 8). All the cells from post-thaw Ad-MSC group cultures remained plastic-adherent and showed fibroblast-like morphology.

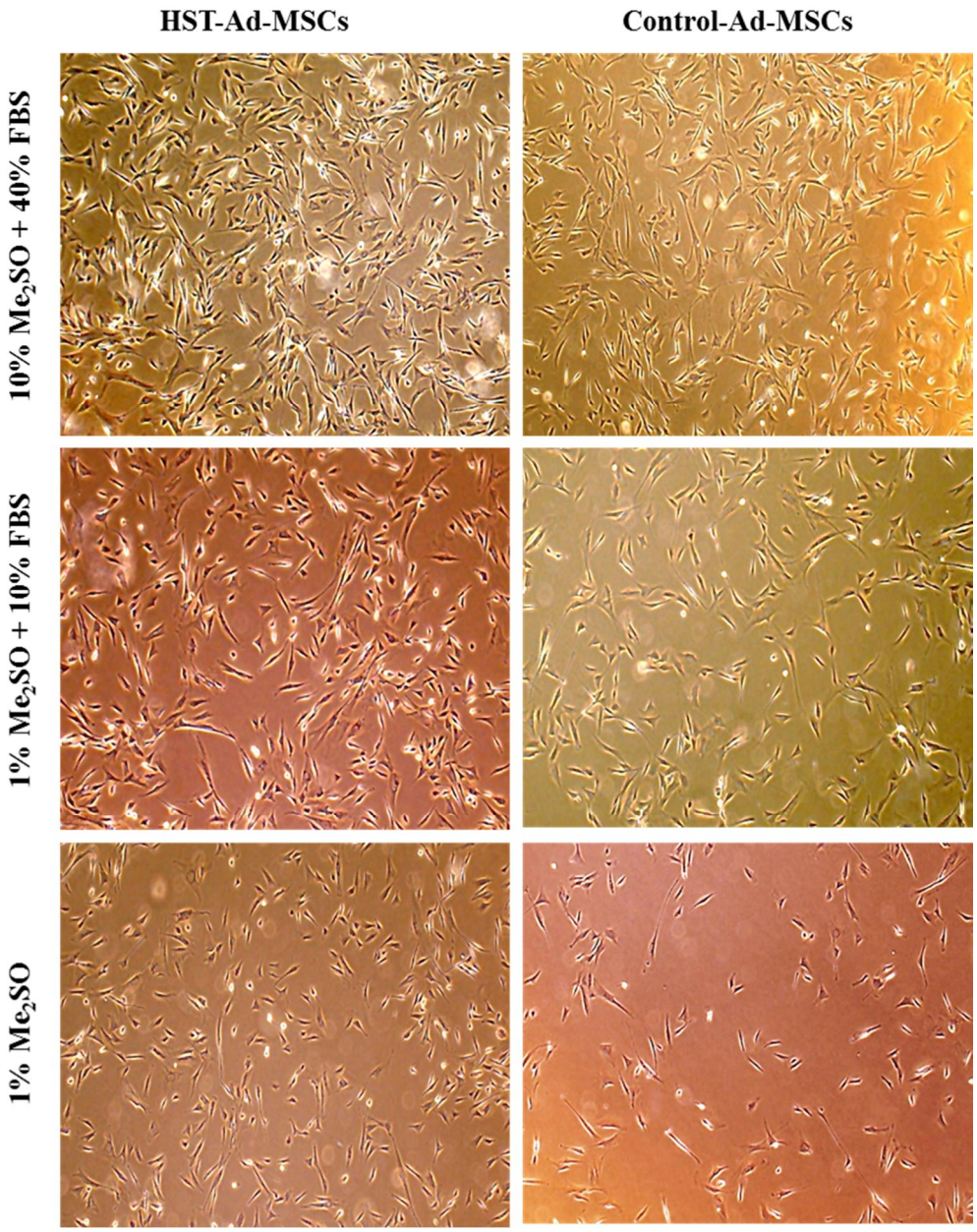

Figure 8 Morphology of cryopreserved stromal cell groups. Post-thaw stromal cells (third passage) 4 d after seeding. The cells from all the groups showed fibroblast-like morphology. Moreover, cell proliferation levels in all the HST groups were significantly ($p < 0.05$) higher compared to their respective control Ad-MSC groups. HST-preconditioned Ad-MSCs stored with 10% Me_2SO + 40% FBS showed the highest ($p < 0.05$) rate of proliferation among all the HST groups as well as control Ad-MSCs after cryopreservation (×40 magnification).

Values for cell viability immediately after thawing of control Ad-MSCs and HST-Ad-MSCs

cryopreserved with 1% Me_2SO, 1% Me_2SO + 10% FBS, and 10% Me_2SO + 40% FBS cryogenic media were 45.0% ± 0.6% and 79.0% ± 0.5%; 72.0% ± 0.6% and 91% ± 0.1%; and 88.0% ± 0.4% and 92.0% ± 0.2%, respectively. HST-Ad-MSCs stored with different cryogenic media showed significantly ($p < 0.05$) higher post-thaw cell viability compared to their respective control groups stored under similar cryogenic conditions (Fig. 9A). Moreover, HST-preconditioned Ad-MSCs stored with 10% Me_2SO + 40% FBS showed the highest ($p < 0.05$) cell viability among all the groups. At 72 h after seeding, the proliferation rates of all HST-Ad-MSC groups stored with different cryogenic media were significantly ($p < 0.05$) higher compared to their respective control Ad-MSCs groups stored under similar cryogenic conditions (Fig. 9B). Moreover, at 24–72 h after seeding, the proliferation rate of the HST group stored with 10% Me_2SO + 40% FBS was significantly ($p < 0.05$) higher compared to the other HST groups (Fig. 9B). Similarly, HST-Ad-MSCs stored with 10% Me_2SO + 40% FBS exhibited significantly ($p < 0.05$) higher antioxidant capacity not only among all the HST groups stored with different cryogenic conditions but also compared to the control Ad-MSC groups (Fig. 9C). This shows that HST pretreated Ad-MSCs stored with 10% Me_2SO + 40% FBS is an optimum condition to preserve the viability, proliferation rate and antioxidant capacity of cells at a significantly ($p < 0.05$) higher level compared to the other groups.

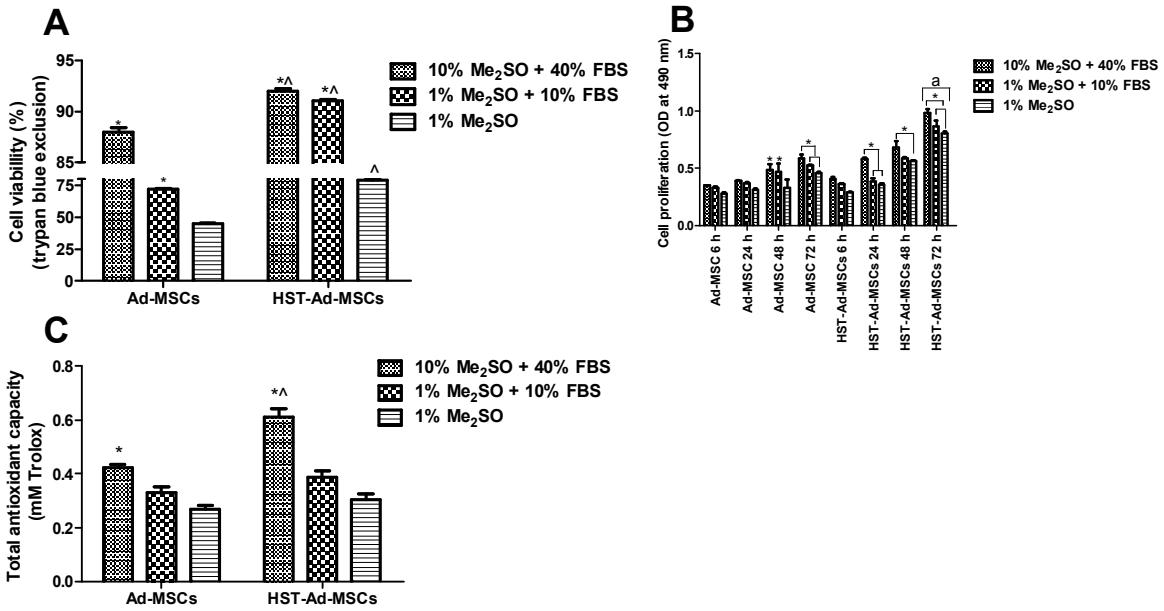

Figure 9 Viability, proliferation rates and anti-oxidation capacity in cryopreserved cultures (A) Post-thaw cell viability levels of control and HST-Ad-MSCs stored under different cryogenic conditions, showing significantly ($p < 0.05$) higher cell viability with HST treatment compared to the respective control groups. (C) HST-Ad-MSCs stored with 10% Me_2SO + 40% FBS showed significantly ($p < 0.05$) higher anti-oxidation capacity than that of the other HST and control groups. *denotes a significant difference among control groups or HST groups. ^denotes a significant difference between control and HST-treated groups stored with similar cryogenic media. (B) The post-thaw proliferation rates of all HST-Ad-MSCs stored under different cryogenic conditions were significantly ($p < 0.05$) higher than those of their respective control groups at 72 h after seeding. Moreover, the proliferation rate of HST-preconditioned Ad-MSCs stored with 10% Me_2SO + 40% FBS was significantly ($p < 0.05$) higher than that of the other HST groups from 24 to 72 h after seeding. *denotes significant ($p < 0.05$) differences among control groups or HST groups; [a] denotes significant ($p < 0.05$) differences between the control and HST groups at 72 h after seeding.

After cryopreservation, *SOX-2* and *NANOG* mRNA expression levels in the HST group cryopreserved with 10% Me_2SO + 40% FBS were the highest ($p < 0.05$) among all the HST

groups as well as the control groups (Fig. 10A and B). However, the mRNA expression level of *OCT-4* in the HST group cryopreserved with 10% Me_2SO + 40% FBS was significantly ($p < 0.05$) higher only compared to its respective control group (Fig. 10C).

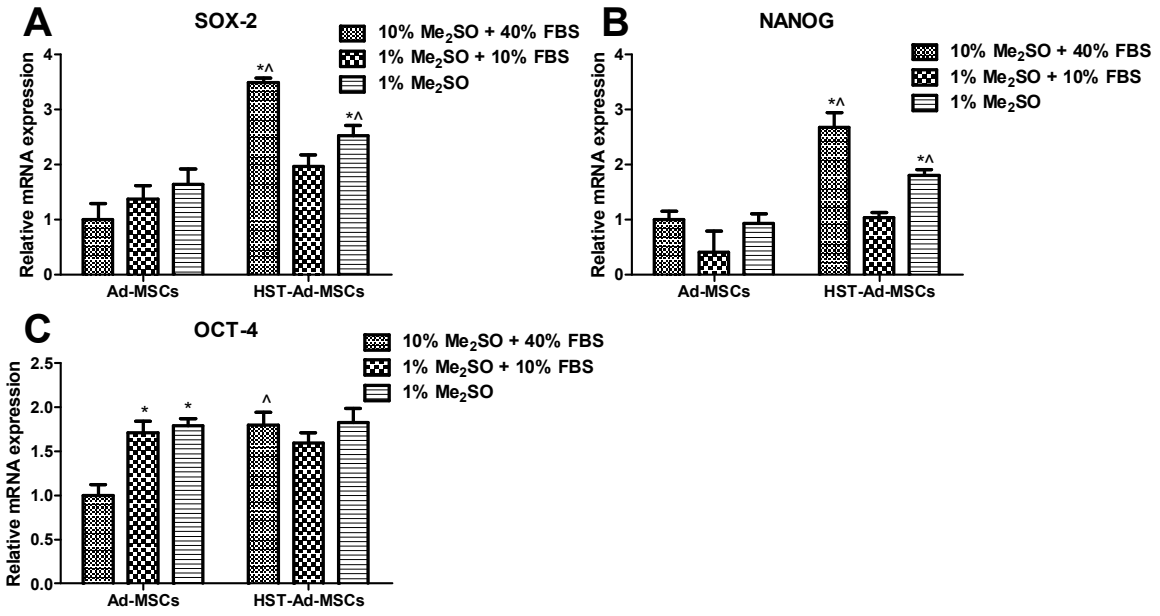

Figure 10 Stemness markers of the cryopreserved groups. (A) HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS showed significantly ($p < 0.05$) higher levels of *SOX-2* and (B) *NANOG* compared to the other HST groups and the control groups (C) HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS showed significantly ($p < 0.05$) higher levels of *OCT-4* expression only when compared to the respective control. *denotes significant differences ($p < 0.05$) among control groups or HST groups. ^denotes significant ($p < 0.05$) differences between the control and HST-treated groups stored with similar cryogenic media.

After cryopreservation, the HST group cryopreserved with 10% Me_2SO + 40% FBS showed significantly ($p < 0.05$) higher mRNA expression levels of *HSP-27* and *HSP-70* compared to the other HST and control groups (Fig. 11A and B). In post-thaw cultures, *HO-1* and copper/zinc-superoxide dismutase (*SOD-1*) mRNA expression levels in HST-Ad-MSCs

cryopreserved with 10% Me_2SO + 40% FBS were significantly ($p < 0.05$) higher than in the other HST groups as well as in the control Ad-MSC groups (Fig. 11C and D).

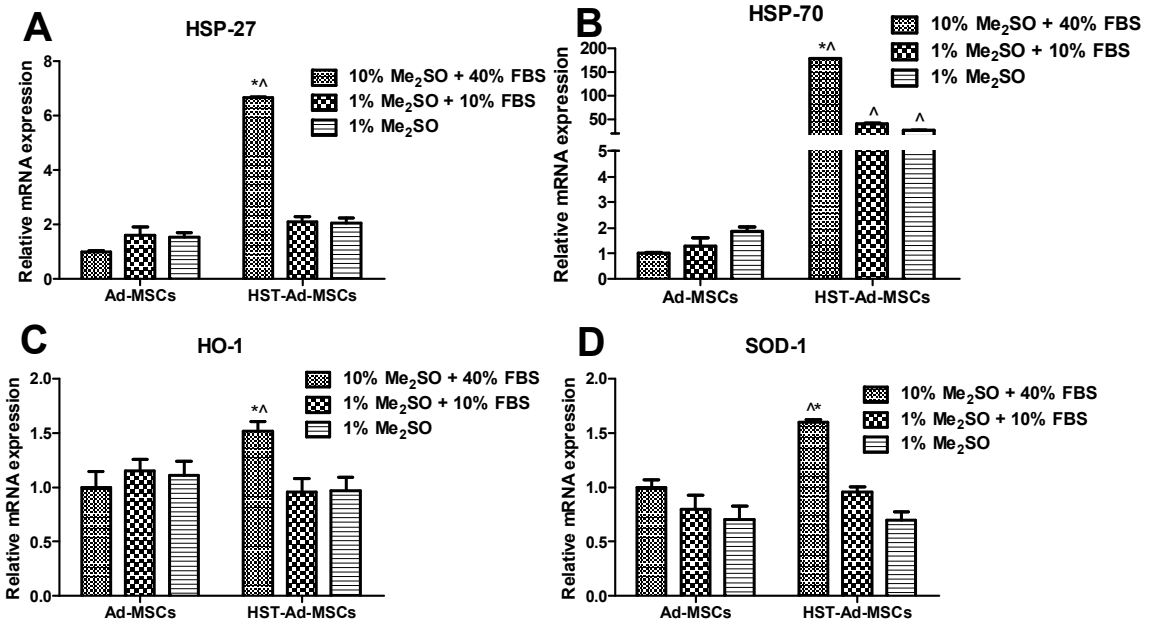

Figure 11 Heat shock proteins mRNA expression of cryopreserved groups. (A) HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS showed significantly ($p < 0.05$) higher *mRNA* levels of *HSP-27* and (B) *HSP-70* compared to all the other groups. (C) HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS showed significantly *($p < 0.05$)* higher *HO-1* and (D) *SOD-1* mRNA expression levels compared to all the other HST groups and control groups. *denotes significant ($p < 0.05$) differences among control groups or HST groups. ^denotes significant ($p < 0.05$) differences between the control and HST groups stored with similar cryogenic media.

After cryopreservation, the overall levels of inflammatory factors (*COX-2* and *IL-6*) in the HST-treated groups were lower compared to their respective control Ad-MSC groups cryopreserved under similar cryogenic conditions (Fig. 12A and B). However, the *IL-10* mRNA expression level in the HST-treated group cryopreserved with 10% Me_2SO + 40% FBS was significantly ($p < 0.05$) higher compared to the other HST groups and the control

groups (Fig. 12C). These results indicate that HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS has better immunosuppressive effects as compared to other groups.

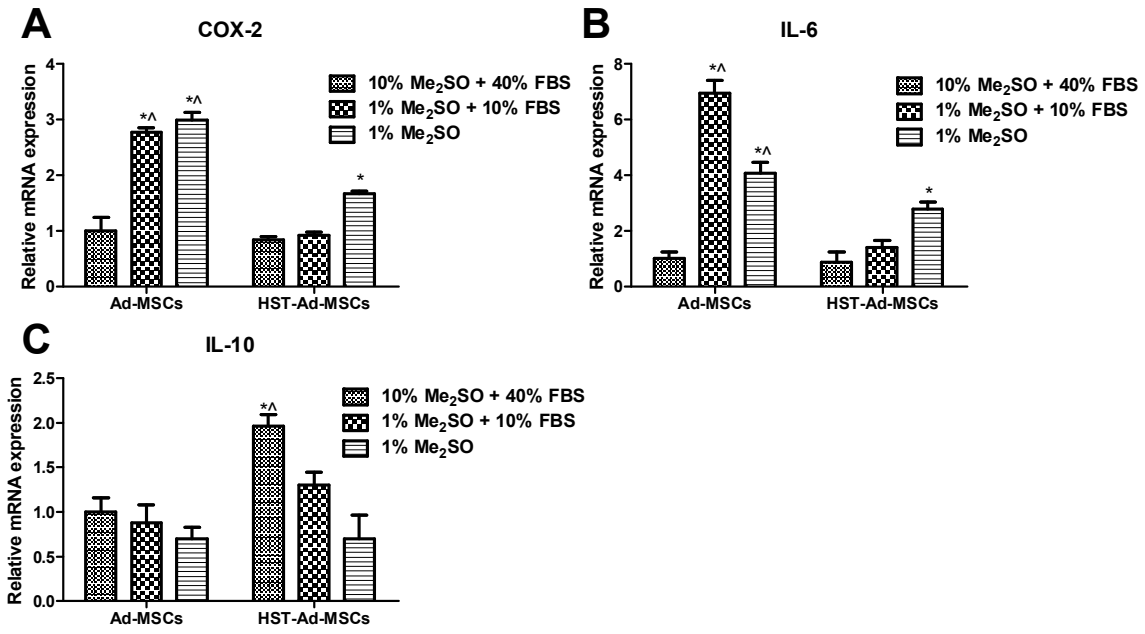

Figure 12 Immunomodulatory factors *mRNA* expression of cryopreserved groups. (A) Overall levels of inflammatory factors *COX-2* and (B) *IL-6* in all the HST groups were lower compared to the respective control groups. (C) However, *IL-10* was significantly ($p < 0.05$) higher in the HST group cryopreserved with 10% Me_2SO + 40% FBS compared to the other HST groups and the control groups. *denotes significant ($p < 0.05$) differences among control groups or HST groups. ^denotes significant ($p < 0.05$) differences between the control and HST- groups stored with similar cryogenic media.

After cryopreservation, the expression level of VEGF mRNA in all HST-Ad-MSC groups cryopreserved under different conditions was significantly ($p < 0.05$) higher compared to their respective control Ad-MSC groups (Fig. 13A). Moreover, the *mRNA* expression levels of *HGF*, platelet-derived growth factor beta (*PDGF*-β), and brain-derived neurotrophic factor (*BDNF*) were significantly ($p < 0.05$) higher in HST-Ad-MSCs cryopreserved with 10%

Me_2SO + 40% FBS compared to other HST groups and the control Ad-MSC group stored under similar cryogenic conditions (Fig. 13B, C and D).

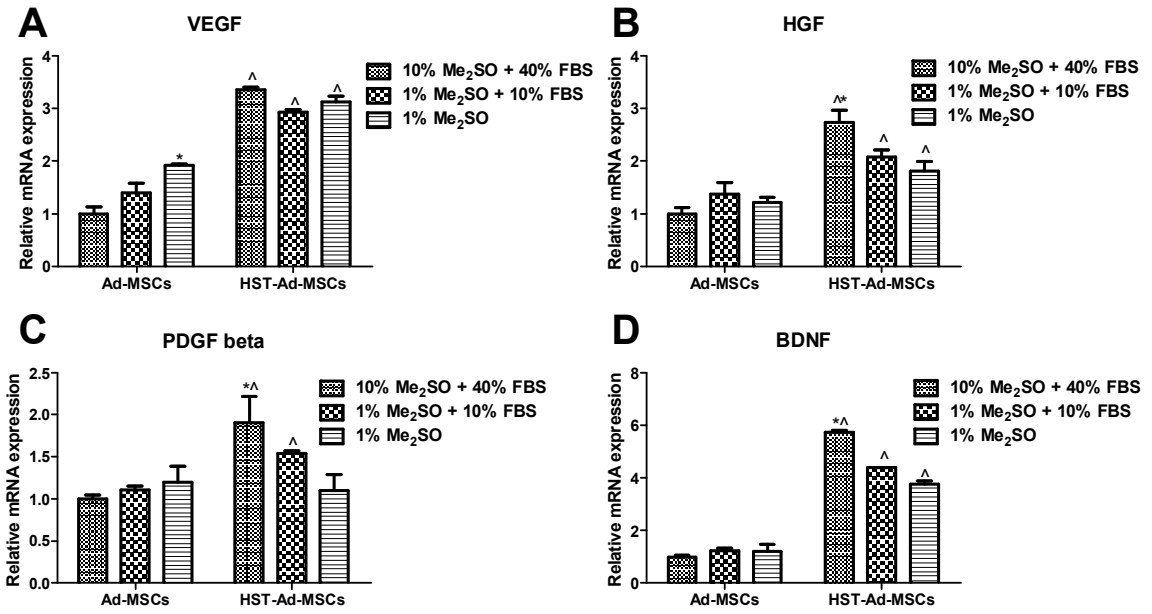

Figure 13 Growth factors *mRNA* expression of cryopreserved groups. (A) In post-thaw groups, all HST-groups cryopreserved under different cryogenic conditions had a significantly ($p < 0.05$) higher level of VEGF expression compared to their respective control Ad-MSCs groups stored under similar cryogenic conditions. (B) *HGF*, (C) *PDGF-β*, and (D) *BDNF* mRNA levels were significantly (*$p < 0.05$)* higher in HST-Ad-MSCs stored with 10% Me_2SO + 40% FBS compared to the other HST groups and the control groups. *denotes significant differences ($p < 0.05$) among control groups or HST groups. ^denotes significant differences ($p < 0.05$) between control and HST groups stored with similar cryogenic media.

There was no significant difference in the mRNA expression levels of *CXCR-4* and *CXCL12* among all the groups after cryopreservation (Fig. 14A and B). However, the *MCP-3 mRNA* expression levels in HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS was significantly ($p < 0.05$) higher compared to all the other groups (Fig. 14C).

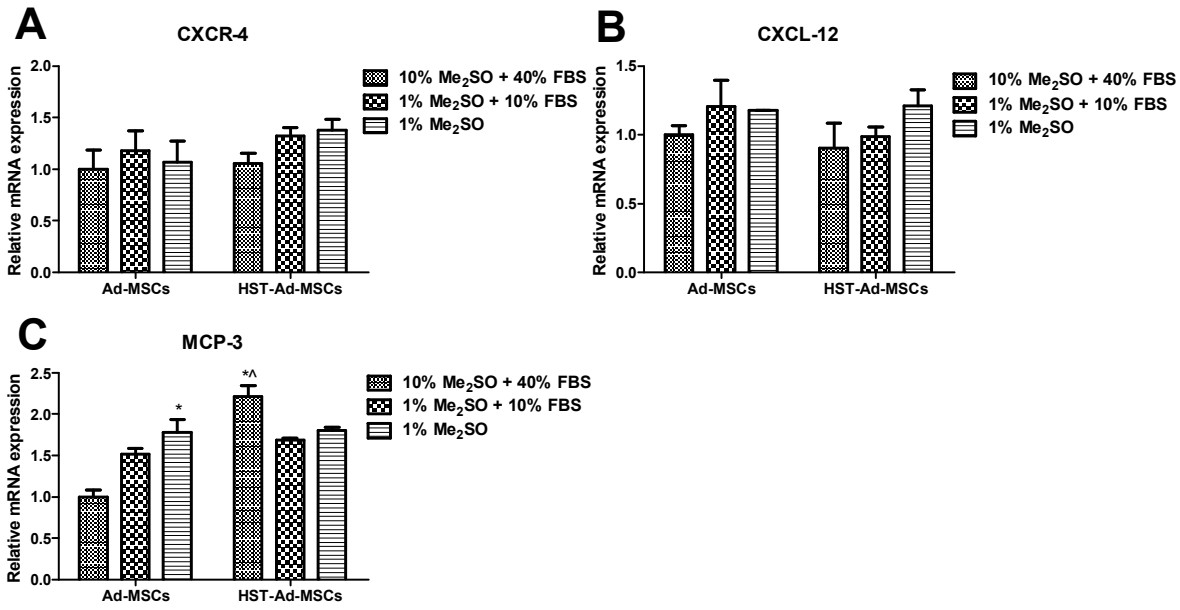

Figure 14 Homing factors *mRNA* expression of cryopreserved groups. (A) In post-thawed culture, there was no significant ($p > 0.05$) difference in the expression level of *CXCR-4* and (B) *CXCL-12* among all cryopreserved groups. (C) However, *MCP-3* of HST-groups stored with 10% Me_2SO + 40% FBS showed significant ($p < 0.05$) increase compared to other groups. *denotes significant differences ($p < 0.05$) among control groups or HST groups. ^denotes significant differences ($p < 0.05$) between control and HST groups stored with similar cryogenic media.

Discussion

In the present study, the morphology of Ad-MSCs did not change after HT or HST treatment, which is in line with other studies (Omori et al., 2014; Safwani et al., 2017). In addition, my results showed that HT + HST treatments together also did not affect the characteristic morphology of Ad-MSCs. Present study results are also consistent with studies showing that cryopreservation does not alter stem cell morphology (Kim et al., 2016). The cells from all fresh Ad-MSCs groups that underwent their respective treatments as well as from all

cryopreserved control and HST groups remained plastic adherent and maintained fibroblast-like morphology.

Previously, Shaik et al. (Shaik et al., 2017) reported that the post-thaw viability and differentiation potential of stem cells was enhanced in FBS-free cryopreserved media with 1% Me_2SO. However, my study showed that post-thaw viability and differentiation potentials were enhanced in HST-Ad-MSCs stored with 10% Me_2SO and 40% FBS as compared to control as well as HST-Ad-MSCs cryopreserved only with 1% Me_2SO. This could be due to the difference in cryopreservation with or without FBS of both studies. As in Shaik et al. study cryopreservation was carried out without FBS while in current study cells were cryopreserved with FBS. Contrary to the findings of Shaik et al., I found that 10% Me_2SO and 40% FBS cryopreserved stromal cells better than 1% Me_2SO only. In fresh culture, HST-Ad-MSCs proliferated more slowly compared to the HT group, likely due to cell death resulting from heat shock treatment (Omori et al., 2014). Omori et al. (Omori et al., 2014) showed that HST treatment (at 43 °C) markedly affected stem cell proliferation until the fourth day after the initial heat shock treatment. However, in this experiment cell death initially could be because of the HST treatment that temporarily reduced stromal cell growth; however, growth started increasing after 48 h and became significantly higher at 72 h compared to control and HT+HST groups (Figure 2B). The difference in results between two studies was likely due to differences in susceptibility to heat shock between the neural stem cells used by Omori et al. (Omori et al., 2014) and the mesenchymal stromal cells used in my study.

In both the fresh and post-thaw cultures, the higher levels of cell viability and proliferation in HST-Ad-MSCs may have resulted from high expression levels of *HSP-27, HSP-70,* and *HO-1*. These heat shock proteins are involved in inhibiting the apoptosis pathway, reducing oxidative stress, and the repairing of misfolded proteins (Shaik et al., 2017). Moreover, the

HST-Ad-MSCs stored with 10% Me_2SO + 40% FBS showed the highest expression levels of *HSP-27, HSP-70,* and *HO-1* of all the post-thaw groups; consequently, this group also showed the highest levels of cell viability and proliferation among all the control and HST groups. The results indicated that viability and proliferation of Ad-MSCs can be efficiently preserved when heat shock-preconditioned Ad-MSCs are cryopreserved in medium containing 10% Me_2SO + 40% FBS.

The total anti-oxidation capacity was significantly higher in the fresh culture of HST-Ad-MSCs compared to the other groups. Moreover, HST-pretreated Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS also presented significantly increased anti-oxidation capacity compared to the other groups. These findings were supported by my RT-qPCR results showing that fresh HST-Ad-MSCs had significantly high levels of HO-1 while post-thaw HST-Ad-MSCs stored with 10% Me_2SO + 40% FBS had significantly high levels of HO-1 and SOD-1. Heme oxygenase 1 (HO-1), also known as HSP-32, is an anti-oxidative enzyme, the upregulation of which enhances cell survival under oxidative stress (Dennery, 2001). While SOD-1 is another antioxidant enzyme that specifically counteracts superoxide anions, and stem cells overexpressing SOD-1 perform better under stress conditions (Wakai et al., 2014). As compared to control significantly higher cell proliferation rates were observed in fresh cultures of HST-Ad-MSCs and in post-thaw cultures of HST-Ad-MSCs stored in cryogenic medium with 10% Me_2SO + 40% FBS, likely due to their increased anti-oxidation capacity; this increase would be expected to reduce the rate of stromal cell death, thus enhancing growth compared to cells with less anti-oxidation capacity. Stem cell anti-oxidation capacity is known to be upregulated by overexpression of HO-1 via transfection by lentivirus (Kim et al., 2016) or adenovirus (Cao et al., 2017) or by the overexpression of SOD-1 (Wakai et al., 2014), which are substantially more complicated events. However, in this study, I came up with a simple and feasible method of enhancing Ad-MSC anti-oxidation

capacity via heat shock treatment. So that Ad-MSCs therapy can be applied immediately in emergency situations.

Several studies have shown that SOX-2, OCT-4, and NANOG play central roles in the stemness of stem cells (Avilion et al., 2003; Mitsui et al., 2003; Rosner et al., 1990), inducing pluripotency when upregulated (Liu et al., 2009; Yoon, Kim, Jung, Paik, & Lee, 2011). In the current study, in a fresh culture, HST treatment significantly increased the stemness marker levels (SOX-2, OCT-4) of Ad-MSCs compared to the other groups. Moreover, HST-Ad-MSCs cryopreserved with 10% Me_2SO + 40% FBS presented significantly higher stemness maker levels (SOX-2, NANOG) compared to the control and other HST groups. Therefore, it is concluded that the stemness of Ad-MSCs is enhanced by HST treatment.

HSP-27 promotes the recovery process in cells by regulating several crucial cellular processes (Ghayour-Mobarhan, Saber, & Ferns, 2012), while HSP-70 has anti-apoptotic and cytoprotective effects (Beere, 2005; Mayer & Bukau, 2005). Therefore, the increase in viability, proliferation rates, and other desired characteristics in the HST groups were after heat shock treatment of fresh or post-thaw cultures may have resulted from the increase in the levels of HSP-27 and HSP-70. As already reported, elevated levels of heat shock proteins reversed the damage caused by stressful conditions through multiple physiological processes, including induction of the apoptotic pathway, repair of denatured and misfolded proteins, and signal transduction (Beere, 2005; Parsell & Lindquist, 1993). Other in vitro treatments such as the addition of recombinant human HSP-70 or transfection of HSP-27 (Andreeva, Zatsepina, Garbuz, Evgen'Ev, & Belyavsky, 2016; Mo et al., 2016) have been used to upregulate the expression of heat shock proteins. However, the heat shock protein induction method used in the current study is convenient and reliable. Therefore, HST helps Ad-MSCs to perform better under stress conditions compared to HT or HT + HST, both in fresh and post-thaw cultures.

COX-2 is a pro-inflammatory enzyme produced in response to intracellular or extracellular stimuli (Williams, Mann, & DuBois, 1999), and it is a therapeutic target for the alleviation of excess inflammatory responses (Masferrer et al., 1994). In contrast, IL-6 links inflammation to malignant transformation by activating the NF-κB pathway (Iliopoulos, Hirsch, & Struhl, 2009). It has also been associated with tumor growth, invasion, and metastasis in human malignancies (Nie, Xue, Sun, Ning, & Zhao, 2014). In this study, I found that HST did not increase the levels of *COX-2* and *IL-6* in fresh cultures of Ad-MSCs. However, in post-thaw cultures, HST-Ad-MSCs stored with 10% Me_2SO + 40% FBS showed decreased levels of inflammatory factors compared to the control groups. This decrease in inflammatory gene expression was likely due to the steep rise in the levels of HSP-70, which is known to exhibit immunosuppressive activity (Stocki & Dickinson, 2012). Moreover, HSP-70 also interfered with the activation of the NF-κB pathway (Yoo et al., 2000). HST significantly increased the levels of *HSP-70* and *IL-10*, both in fresh and post-thaw Ad-MSCs. These results agreed with other studies showing that IL-10 increases in a dose-dependent manner with increasing levels of HSP-70 (Stocki & Dickinson, 2012). My data also showed that HST treatment increased the levels of *HGF*, which is reported to promote IL-10 and reduce IL-6 levels (Wang et al., 2017). Therefore, the increase in IL-10 and decrease in IL-6 mRNA levels may have also resulted from the increase in *HGF* levels in response to HST treatment. This immunosuppressive effect of heat shock treatment on Ad-MSCs could be very useful for in-vivo therapy to produce immunosuppression.

VEGF plays a crucial role in wound healing by increasing microvessel permeability, promoting endothelial cell growth, and facilitating endothelial cell migration through the extracellular matrix (Enestvedt et al., 2008). In contrast, HGF reduces endothelial apoptosis and induces endothelial cell proliferation. It also reduces IL-6 and increases IL-10 production (Wang et al., 2017). PDGF, a potent mitogen of cells of mesenchymal origin (including stem

cells) (Pierce, Mustoe, Altrock, Deuel, & Thomason, 1991), increases VEGF production in stem cells in a dose-dependent manner. It also activates numerous pathways important for regulating cell growth, proliferation, and differentiation (Andrae, Gallini, & Betsholtz, 2008; Razmara, Heldin, & Lennartsson, 2013). BDNF is the most prevalent neurotrophic factor in adult brains and is important for neuronal survival and activity (Wollen, 2010). The levels of all these growth hormones increased after HST to levels higher than those of other treatments in both fresh and post-thaw cultures. Therefore, compared to other treatments, HST treatment of Ad-MSCs, either fresh or cryopreserved, has the potential to improve the outcome of tissue regeneration as part of regenerative medicine. Further studies are required to elucidate the exact mechanisms by which heat shock treatment upregulates these growth hormones.

CXCL12, CXCR-4, and MCP-3 have been implicated as important regulators of migration or homing of stem cells (Guo, Hangoc, Bian, Pelus, & Broxmeyer, 2005; Schenk et al., 2007). Previously, the upregulation of CXCR-4 and MCP-3 was shown to increase stem cell migration to a site of injury (Cheng et al., 2008; Schenk et al., 2007). Therefore, fresh HST-Ad-MSCs are likely to exhibit improved homing capacity compared to other groups. However, in post-thaw HST and control groups, I found no significant difference in the levels of homing factors, except for MCP-3. MCP-3 has been reported to recruit stem cells to sites of injured tissue (Schenk et al., 2007); therefore, local application of post-thawed HST-Ad-MSCs may be helpful in recruiting stem cells to enhance healing or remolding.

Cryopreserved cells are more convenient to use than fresh cells, but cryopreservation also has some disadvantages. In the present study, I showed that heat shock can be used to overcome the drawbacks of cryopreservation to a greater extent than hypoxia or hypoxia + heat shock treatment. The importance of my research is in the integration of cryopreservation with heat shock treatment, which opens the possibility of generating cells that can be used immediately in a clinical setting.

Conclusion

In conclusion, heat shock treatment enhanced the viability and antioxidant capacity, as well as the levels of anti-stress, anti-inflammatory, and growth factors of adipose-derived mesenchymal stromal cells to levels that were higher than those observed with other prescribed treatments. This applies not only to fresh cultures but also to those that have been cryopreserved.

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Korean Abstract (논문 초록)

Heat shock protein 활성화 처리가 신선 및 동결 후 개 중간엽 줄기 세포의 생물학적 특성의 증진 효과

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동종 지방유래 줄기세포(Ad-MSCs)는 항 산화와 항 염증 특성을 가지고 있는 세포 치료제의 좋은 자원이다. 동결 Ad-MSC 는 세포치료제로서 즉시 사용될 수 있지만, 동결보존은 특히 세포 생존성과 산화 방지 능력 측면에서 Ad-MSCs 에 해롭다. 따라서 줄기세포의 임상적용을 활성화하기 위해서는 동결보존세포의 해동 후 세포 특성을 개선하기 위한 연구가 요구되고 있다. 첫번째 연구에서는, Heat shock protein (HSP) 활성화처치, 5% 저산소 (Hypoxia)하에서의 배양 또는 HSP 활성화 처치 + Hypoxia 하 배양에 대한 세포 특성의 변화를 조사하였다. 그 결과, 다른 처치법들과 비교했을 때, HSP 활성화처치는 항 산화 능, 성장 인자 및 증식과 관련된 전반적인 세포 특성을 크게 향상시켰다. 두번째 연구에서는 HSP 활성화처치 세포에 대한

최선의 냉동 보존 방법을 알아보기 위하여 냉동제인 Dimethyl sulfoxide (Me_2SO)와 소 태아혈청 (FBS)의 조합을 비교하였다. 1% Me_2SO + 10% FBS 또는 1% Me_2SO 만으로 동결보존한 것과 비교했을 때 10%의 Me_2SO + 40% FBS 으로 보존된 HSP 활성화처치 세포가 생존성, 증식력, 항 산화능 및 미분화능의 개선뿐만 아니라 HSP, 성장인자 및 항 염증인자의 유의미한 수준의 향상도 보여주었다. 이상의 결과를 고려할 때 줄기세포의 HSP 활성화 처리는 동결 후의 생물학적 특성을 강화시킬 수 있는 유의미한 방법이라 생각된다.

주요어: 지방유래 줄기세포, 동결보존, Heat shock protein 활성화 처리, 저산소하 배양

학번: 2017-21476